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Research Article

RESEARCH ARTICLE

The impact of nectar chemical features on phenotypic variation in two related nectar yeasts

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One sentence summary: The variation of nectar chemistry across plant species affects nectar consumers, such as pollinators. We have found here that nectar chemistry also affects nectarivorous yeasts. There is a large variation in yeast phenotype according to the host plant from which they are isolated, and some nectar chemical traits, such as sugar concentration and fructose content, help to explain that phenotypic landscape.

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ABSTRACT

Floral nectars become easily colonized by microbes, most often species of the ascomycetous yeast genus *Metschnikowia*. Although it is known that nectar composition can vary tremendously among plant species, most probably corresponding to the nutritional requirements of their main pollinators, far less is known about how variation in nectar chemistry affects intraspecific variation in nectarivorous yeasts. Because variation in nectar traits probably affects growth and abundance of nectar yeasts, nectar yeasts can be expected to display large phenotypic variation in order to cope with varying nectar conditions. To test this hypothesis, we related variation in the phenotypic landscape of a vast collection of nectar-living yeast isolates from two *Metschnikowia* species (*M. reukaufii* and *M. gruessii*) to nectar chemical traits using non-linear redundancy analyses. Nectar yeasts were collected from 19 plant species from different plant families to include as much variation in nectar chemical traits as possible. As expected, nectar yeasts displayed large variation in phenotypic traits, particularly in traits related to growth performance in carbon sources and inhibitors, which was significantly related to the host plant from which they were isolated. Total sugar concentration and relative fructose content significantly explained the observed variation in the phenotypic profile of the investigated yeast species, indicating that sugar concentration and composition are the key traits that affect phenotypic variation in nectarivorous yeasts.

Keywords: floral nectar; high-throughput; *Metschnikowia*; phenotypic landscape

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INTRODUCTION

Nectar is the most common reward that plants offer to their mutualistic counterparts (Simpson and Neff 1983). Although some nectar traits (e.g. sugar concentration) are subject to environmental variation, nectar characteristics of pristine nectar are genetically determined and bear a strong phylogenetic imprint (Nicolson and Thornburg 2007). As a result, several nectar traits, including sugar concentration, sugar composition, pH and amino acid content, have been shown to vary widely among species (Herrera 2014; Herrera, Pozo and Bazaga 2012; Herrera, Pozo and Bazaga 2014). Moreover, interspecific variation in nectar traits is strongly related to pollinator guilds (Baker and Baker 1983; Petanidou 2005; Petanidou *et al.* 2006; Nicolson 2007). For example, the spectrum of flower-visiting animals and nectar consumers, many of which act as pollinators, is strongly determined by nectar sugar concentration (total sugars) and composition (ratio of sucrose and its monosaccharides fructose and glucose). The strong association between nectar traits and pollinator guilds can be best explained from the animal perspective, since plant products that are secreted to attract animal pollinators should match their nutritional requirements, or at least be in concordance with their physiological constraints (Nicolson 2007). For instance, the high frequency of hexose-rich nectar has been related to the lack of invertase in many nectar feeders (Napier *et al.* 2013).

Most nectars are, however, not exclusively consumed by mutualistic partners and in most floral nectars simple microbial communities have been shown also to exploit this resource (Herrera, Garcia and Perez 2008: Pozo, Herrera and Bazaga 2011; Peay, Belisle and Fukami 2012). In most cases, nectar microbial communities are dominated by yeasts, which can reach very high densities in floral nectar (up to 10^5 cells mm^{-3}) (Herrera *et al.* 2009). Quite often, yeasts and bacteria co-inhabit floral nectaries (Fridman *et al.* 2012; Álvarez-Pérez, Herrera and de Vega 2013). Irrespective of the host plant species, the most common nectarivorous yeasts are members of the genus *Metschnikowia* (Pozo, Herrera and Bazaga 2011). Many species of this genus appear to be specialized in the flower–insect interphase (Lachance and Starmer 1998; Lachance *et al.* 2011) and are transferred from flower to flower by pollinators, most often insects (de Vega, Herrera and Johnson 2009; Herrera *et al.* 2009), but sometimes also by birds (Belisle, Peay and Fukami 2012) or ants (de Vega and Herrera 2013). The high preponderance of *Metschnikowia* in floral nectar has been related to their ability to exploit a wide diversity of variable resources efficiently (Herrera, Pozo and Bazaga 2012). Nevertheless, intrinsic host plant features may impose severe constraints on nectar colonization (Herrera, Pozo and Bazaga 2012; Herrera, Pozo and Bazaga 2014), and favour or be detrimental to the establishment of certain strains (Pozo, Lachance and Herrera 2012).

While for animal pollinators floral nectar merely represents a feeding resource, for nectar-dwelling microorganisms nectar is the vital environment in which they have to survive. Natural variation in the chemical properties of floral nectar should therefore impose strong selective forces that might have a large influence on the phenotypic landscape of nectarivorous yeasts. High sugar concentration has been shown to represent a major constraint for microbial growth, as it decreases water activity and could result in cell rupture and death (Lievens *et al.* 2015). Besides sugar concentration, sugar composition may also represent an important environmental constraint, as yeast performance largely depends on the carbon sources that are available (Kurtzman, Fell and Boekhout 2011; Herrera, Pozo and Bazaga 2012). From the perspective of nectar-living organisms, this implies that floral nectars produced by different host plants are not equivalent in terms of their quality as microhabitats. As a result, natural plant communities should be seen as highly heterogeneous environments that determine the specific conditions where a certain phenotype proliferates. Therefore, it can be hypothesized that nectar yeasts may be organized in phenotypic clusters, which are highly determined by the host plant species from which they were isolated (Herrera, Pozo and Bazaga 2012; Pozo, Lachance and Herrera 2012).

To test this hypothesis, a large collection of *M. reukaufii* and *M. gruessii* strains from different origins was subjected to a wide variety of phenotypic assays that are relevant in the context of nectar-associated yeasts. Preliminary research using a limited number of yeast isolates has shown that two co-occurring species of the genus *Metschnikowia* (*M. reukaufii* and *M. gruessii*) exhibited considerable interspecific divergence (M.I. Pozo *et al.*, unpublished results), most probably explaining their coexistence in floral nectar. Moreover, the two species also displayed substantial intraspecific variation in phenotypic traits, but it was unclear to what extent this intraspecific variation was mediated by the host plant species, and which nectar traits were most important in determining phenotypic variation. To address these questions, we significantly extended our existing yeast collection to include yeast isolates from additional plant species. More specifically, we determined natural variation in floral nectar properties across 19 plant species and tested whether intraspecific phenotypic variation was related to host plant species. Secondly, we tested whether host-specific nectar properties were related to phenotypic variation in nectar yeast isolates.

MATERIALS AND METHODS

Study species

Metschnikowia reukaufii and *M. gruessii* are ascomycetous yeasts belonging to the Saccharomycetales clade. Both species are closely associated with floral surfaces, floral nectar and flower-visiting insects such as bumble-bees (Brysch-Herzberg 2004). The yeasts are transferred to the flowers by foraging insects, most often solitary and social bees (Brysch-Herzberg 2004; Herrera *et al.* 2010; Pozo, Lachance and Herrera 2012). Vegetative cells found in flowers are diploid and proliferate profusely by multilateral budding, reaching densities in floral nectar $>5 \times 10^4$ cells mm^{-3} within 2–4 days after colonization.

Nectar chemistry

To assess variation in total sugar concentration, sugar composition and nectar pH, pristine (i.e. free of microbes) nectar samples were collected from 19 different host plant species belonging to seven plant families. All samples were collected in the Sierra de Cazorla-Segura-Las Villas Natural Park, a well preserved mountainous region in south-eastern Spain. Between two and 23 individual plants per plant species were selected at the beginning of the blooming season, depending on the availability of well-preserved plant populations in the region (Table 1). To avoid microbial nectar contamination, visiting insects were excluded from flowers by bagging the inflorescences with a 0.1 mm^2 mesh (Pozo, Lachance and Herrera 2012). Open flowers, if any, were removed before bagging. Every 2 days, plants were checked in order to record open flowers. At an intermediate flower age (depending on the plant species), flowers were collected in the field

Table 1. Summary statistics of nectar properties by plant species.

				Sugar concentration (% sugars in 100 g)			Sugar composition (% of total sugars, mean ± SE)			pH		
Plant family	Plant species	Plants, *n*	Samples, *n*	Mean ± SE	Max	Min	% Suc	% Fru	% Glu	Mean ± SE	Max	Min
Caprifoliaceae	*Lonicera etrusca*	5	5	17.9 ± 0.0	17.9	17.9	64.6 ± 1.6	12.7 ± 0.1	22.7 ± 0.7	6.5 ± 0.0	6.5	6.5
Caprifoliaceae	*Lonicera implexa*	5	12	26.0 ± 1.4	33.5	14.7	63.5 ± 2.9	14.2 ± 1.3	22.3 ± 1.6	6.7 ± 0.1	7	6
Fabaceae	*Tetragonolobus maritimum*	3	8	21.3 ± 2.4	29.1	7.2	69.6 ± 1.9	13.5 ± 0.8	16.9 ± 1.0	6.6 ± 0.2	7	6
Fabaceae	*Vicia onobrychioides*	3	4	31.1 ± 4.1	37.5	19.3	54.2 ± 5.6	19.6 ± 2.4	26.2 ± 3.5	6.5 ± 0.3	7	6
Fabaceae	*Vicia villosa*	4	5	35.5 ± 2.5	41.5	28.2	56.0 ± 4.8	20.1 ± 2.0	23.9 ± 2.8	6.8 ± 0.2	7	6
Iridaceae	*Gladiolus illyricus*	5	6	13.1 ± 3.5	17.8	6.3	51.3 ± 3.3	18.0 ± 1.4	30.7 ± 1.9	8.0 ± 0.0	8	8
Iridaceae	*Iris foetidissima*	8	8	27.6 ± 2.0	35.5	18.2	94.1 ± 1.2	2.3 ± 0.5	3.6 ± 0.7	6.8 ± 0.1	7	6.5
Iridaceae	*Iris pseudacorus*	4	6	16.8 ± 2.5	27.7	10.9	71.4 ± 5.3	10.0 ± 2.2	18.6 ± 3.1	7.1 ± 0.4	8	6
Lamiaceae	*Marrubium supinum*	2	2	26.9 ± 13.4	40.3	13.6	43.6 ± 1.7	29.7 ± 0.4	26.7 ± 1.3	6.8 ± 0.3	7	6.5
Lamiaceae	*Teucrium pseudochamaepytis*	9	12	20.9 ± 2.0	28.7	2.9	53.2 ± 2.7	20.2 ± 1.4	26.6 ± 1.4	6.8 ± 0.1	7	6
Plantaginaceae	*Antirhinum australe*	3	4	18.8 ± 7.6	36.3	2.7	78.2 ± 6.4	12.5 ± 4.7	9.4 ± 1.9	6.1 ± 0.4	7	5
Plantaginaceae	*Digitalis obscura*	5	5	20.4 ± 4.1	28.6	5.8	63.9 ± 2.7	31.7 ± 3.0	4.4 ± 0.4	6.0 ± 0.0	6	6
Plantaginaceae	*Linaria aeruginea*	2	10	40.4 ± 0.8	44.0	36.3	70.2 ± 2.0	13.7 ± 0.9	16.1 ± 1.2	6.7 ± 0.1	7	6
Plantaginaceae	*Linaria lilacina*	2	4	26.5 ± 8.7	44.9	6.3	78.8 ± 1.7	7.4 ± 0.2	13.8 ± 1.5	5.9 ± 0.3	6.5	5
Plantaginaceae	*Linaria verticillata anticaria*	3	5	32.7 ± 5.8	46.8	12.0	74.8 ± 2.3	10.4 ± 1.1	14.8 ± 1.2	6.8 ± 0.2	7	6
Ranunculaceae	*Aquilegia pyrenaica*	8	42	28.7 ± 8.3	47.2	11.8	98.4 ± 0.9	0.6 ± 0.6	1.0 ± 0.5	7.8 ± 0.9	9.3	6.2
Ranunculaceae	*Aquilegia vulgaris*	10	20	29.6 ± 1.1	37.8	21.9	96.0 ± 0.9	1.5 ± 0.3	2.5 ± 0.6	7.1 ± 0.0	7.5	7
Ranunculaceae	*Helleborus foetidus*	10	39	39.7 ± 9.3	73.7	20.0	97.3 ± 0.7	2.3 ± 0.6	0.4 ± 0.1	8.2 ± 1.0	9	5
Solanaceae	*Atropa baetica*[a]	23	102	68.0 ± 1.4	93.1	34.0	43.1 ± 8.4	12.6 ± 3.8	12.3 ± 3.8	6.3 ± 0.5	6.8	5.8

[a]Nectar samples were not pooled in this plant species.

very early in the morning and carried inside a cooler to the lab where flowers were immediately processed.

For each flower, nectar was extracted with sterile 5 μl calibrated glass microcapillaries (IntraMARK BLAUBRAND, Wertheim, Germany). Because a volume of up to 5 μl per sample was needed to assess sterility, sugar concentration, nectar pH and sugar composition, up to 10 flowers per plant were collected for plants with small flowers and smaller volumes of nectar and pooled afterwards. In this way, 4–102 nectar samples were obtained for each plant species (see Table 1). Once the necessary amount of nectar was collected, the total volume was divided in a sequential workflow. First, we ensured that nectar samples were not contaminated by yeasts. To do so, 4.5 μl of the staining product Lactophenol cotton blue was added to 0.5 μl of each nectar sample and inspected under the microscope using ×40 magnification (Herrera *et al*. 2009). Once sterility was confirmed, nectar was deposited on a hand refractometer (Bellingham & Stanley Ltd, Tunbridge Wells, UK) to measure total sugar concentration, which was expressed as percentage sucrose equivalents (grams of sucrose per 100 g of solution). Nectar pH was estimated to the nearest 0.5 unit using a universal pH indicator (range 1–14; Panreac, VWR). The composition of the main nectar sugars was determined by high-performance anion-exchange chromatography (Thermo Fisher Scientific Dionex, Sunnyvale, CA, USA) with pulsed amperometric detection (HPAEC-PAD). To this end, a part of the previously gathered nectar sample was stored in Whatman No. 1 filter-paper wicks (Dafni 1992; Galetto and Bernardello 2005). Wicks were dried with silica gel and stored at room temperature prior to analysis. Nectar was recovered from the filter paper by static elution at 5°C with 500 ml of milliQ water during 24 h. Once recovered, the samples were filtered by a polyvinylidene fluoride membrane 0.45 μm filter. In the particular case of *Helleborus foetidus* and *Atropa baetica*, samples for HPAEC-PAD analyses were stored using capillaries of 1 μl that were diluted in 500 μl of milliQ water and kept at –80°C. Prior to analysis, nectar samples were diluted with milliQ water and analysed as outlined earlier (Vergauwen, Van den Ende and Van Laere 2000; Herrera, Pérez and Alonso 2006). The concentrations of the different sugars in each analysed sample were estimated by comparing the area under the chromatogram peaks with standards using Chromeleon software (Thermo Fisher Scientific Dionex). The percentage of fructose, glucose and sucrose was determined and expressed as w/w of total sugars determined by HPAEC-PAD. Only sucrose, glucose and fructose appeared in the analyses.

Yeast sampling

Yeasts were isolated from several nectar samples that were collected over different years (2008, 2009 and 2013) from the same 19 plant species. Yeasts were isolated using the methods described in Pozo, Herrera and Bazaga (2011). Briefly, isolation consisted of spread-plating nectar onto Yeast Extract Glucose Chloramphenicol (YGC hereafter; Sigma Aldrich) agar plates, followed by incubation, purification and identification of the obtained isolates (one isolate per colony morphotype). In total, this resulted in 600 *M. reukaufii* and 76 *M. gruessii* isolates (Supplementary Table S1). Divergence in sample size is due to the higher frequency of *M. reukaufii* in some plant species that were investigated, such as *Helleborus foetidus*. Isolates were identified on the basis of both morphological characteristics and two-way sequencing of the variable D1/D2 domain of the large subunit (26S) rRNA gene using primers NL1 and NL4 (O'Donnell 1993). Consensus sequences were compared with the type strain sequences (CBS 7657 for *M. gruessii* and CBS 5834 for *M. reukaufii*) obtained from GenBank (accession nos U45737 and U44825, respectively). The isolates were stored in Microbank™ preservation system vials at –80°C. For this study, isolates were transferred to 96-well plates with 40% glycerol in sterile deionized water in which they were stored at –80°C until further use.

Phenotypic profiling and preparation of media

For this study, 46 phenotypic tests were used, including standard tests used in yeast systematics (Kurtzman, Fell and Boekhout 2011) and a number of tests developed for phenotypic characterization of nectar yeasts [see Pozo, Lachance and Herrera (2012) for further details]. Selected assays represented tests related to assimilation of carbon (27 parameters) and nitrogen (3), osmotolerance (3), halotolerance (2), resistance to inhibitors (8), growth on a high pH medium, growth on a vitamin-free medium and hydrolysis of Tween-80 (Supplementary Table S2). Solute concentrations are presented on a weight to weight percent basis.

For inhibitor and halotolerance tests, YM agar (0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1% glucose, 2% agar) was used as basic medium, supplemented with the test compound (for tested concentrations, see Supplementary Table S2). Osmotolerance was evaluated using 50% glucose, 50% fructose and 50% sucrose, supplemented with 1% yeast extract. For halotolerance, we used two concentrations of NaCl (5% and 10%), whereas for carbon source assimilation we tested 1% of each compound in Yeast Nitrogen Base (YNB), with YNB-Glucose 1% and YNB as the positive and negative reference plates, respectively. To test nitrogen assimilation, we tested 0.06% of every selected nitrogen source in Yeast Carbon Base (YCB), while growth in YCB medium without added nitrogen source was used as reference.

All ingredients used in the preparation of the test media were purchased from Sigma Aldrich. Carbon and nitrogen sources were autoclaved separately to avoid Maillard reactions and formation of inhibitory compounds, and mixed when the temperature had decreased to ~50°C. Some ingredients such as ethanol, deoxycholic acid, Na_2CO_3 and HCl were added after autoclaving.

Phenotypic profiling

In order to maximize throughput and reproducibility, a high-density array robot (Singer ROTOR HDA, Singer Instruments, Roadwater, Somerset, UK) was used to produce and replicate the selected yeast strains on the different test media. More specifically, the 96-well plates containing the stored isolates were first thawed, spotted on YGC agar (2.0% glucose, 0.5% yeast extract, 2.0% agar and 0.01% chloramphenicol) and incubated at 24°C for 2 days. Next, 96-well plates containing 150 μl of medium YM broth (YMB) (1.0% glucose, 0.5% peptone, 0.3% malt extract and 0.3% yeast extract) in each well were inoculated with the strains using the robot and incubated overnight at 24°C on a microplate shaking platform (Heidolph, Germany) at 900 rpm. Then, the optical density at 600 nm (OD_{600}) of all wells was measured using a microplate reader (Molecular Devices, USA), yielding values that were consistently 1, with 0.9 and 1.9 being the most disparate values obtained. In those cases, the cell density was manually adjusted to OD_{600} ~1.0 in a second 96-well microtitre plate using sterile deionized water. This plate was used as the source plate for spotting those cell suspensions immediately onto the test media plates with the HDA rotor. After spotting, all agar plates were sealed using parafilm (Pechiney Plastic Packaging Company, USA) and incubated at 24°C. After 2 days of incubation,

all plates were scanned using a high-definition scanner (Seiko Epson, Nagano, Japan). To evaluate osmotolerance, plates were incubated for 12 days and regularly scanned (after 2, 4 and 12 days

To obtain the phenotypic profiles of the strains, the colony area of each strain on each test plate was measured. Scanned images of the test plates were processed using ImageJ (Abramoff, Magalhaes and Ram 2004), combined with the ScreenMill software especially developed for this purpose (Dittmar, Reid and Rothstein 2010). For carbon and nitrogen assimilation, the relative growth after 2 days of incubation was calculated as the growth in a certain condition ('test condition') relative to the growth on the control medium. Strains yielding colony areas of <100 pixels on the reference YNB-Glucose plate after 2 days of incubation were discarded from the analysis (30 *M. reukaufii* isolates were discarded from the initial $n = 600$). To evaluate osmotolerance, the growth rate was calculated as $r = (\ln A_f - \ln A_0)/t$, where A_0 is the initial colony area, A_f is the final colony area after growth and t is the number of days between the first and last measurement. Growth was assessed after $t = 10$ days for sucrose and $t = 8$ days for glucose and fructose. As resistance to inhibitors was independent of the reference plates (data not shown), we recorded the actual growth there.

Statistical analyses

Multivariate analyses of variance were used to compare phenotypic profiles between isolates of the two yeast species separately. Those isolates were isolated from different plant species (19 plant species for *M. reukaufii* and 9 for *M. gruessii*) and plant families (7 and 6, respectively). As the data were not normally distributed and therefore did not fit to the assumptions of a classic multivariate analysis of variance (MANOVA), a non-parametric variant (PERMANOVA) implemented in the 'adonis' (multivariate analysis of variance based on dissimilarity matrices) function in the R package 'vegan' (Anderson 2001; Oksanen *et al.* 2013; Majetic, Levin and Raguso 2014) was used. In this analysis, the impact of sampling year was treated as a random effect. For all tests, dissimilarity matrices between isolates were calculated based on the Bray–Curtis distance using the 'vegdist' function from the 'vegan' package, except when evaluating the osmotolerance data set, where Gower distances were used. Due to small sample sizes for some host plant species, four and 12 plant species were retained in the analyses for *M. gruessii* and *M. reukaufii*, respectively. Additionally, separate PERMANOVAs were performed without the host species *H. foetidus* for the *M. reukaufii* data set, due to the fact that this plant species was over-represented in that data set.

Univariate analyses (PROC UNIVARIATE, SAS Institute Inc., 2012) indicated that variables related to nectar chemistry (sugar concentration, pH and the relative percentages of fructose, glucose and sucrose) were not normally distributed; therefore, non-parametric Wilcoxon and Kruskal–Wallis tests (NPAR1WAY, SAS Institute Inc., 2012) were used to see whether nectar variables differed between plant species or between plant families. Hierarchical variance partitions using restricted maximum likelihood (REML) were performed to assess the percentage variation between plant families and between plant species within families.

We used automatic stepwise model building procedures to assess the amount of intraspecific variation in the phenotypic data in two yeast species that can be attributed to the nectar chemical data. A forward stepwise approach was applied as implemented in the 'ordistep' function (vegan package) in R: variables were added step by step into the model and, at each turn, the variable with the highest portion of variance explained was kept, until no other variable significantly increased the portion of the variance explained ($P < 0.05$). The significance of the variables was tested by 9999 permutations. We did these analyses for the overall set of test parameters, and also for parameters grouped by test type separately (see Supplementary Table S2): carbon sources, nitrogen sources, water activity, hydrolysis, pH and vitamin test, and inhibitors. The whole variable selection procedure was run for each yeast species and parameter type separately, leading to different sets of variables for the two species and parameters.

Finally, to visualize general trends in phenotypic variation, results from non-metric multidimensional scaling (nMDS) analyses (Clarke and Warwick 2001; Majetic, Levin and Raguso 2014) were plotted on top of interpolated and smoothed environmental surfaces. nMDS analyses were conducted for each yeast species and parameter type separately, based on Bray–Curtis dissimilarities, except for low water activity parameters, where the Gower distance was used. For the set of statistically significant nectar variables selected earlier, two-dimensional thinplate spline surfaces were fitted between nectar variables and phenotypic traits using the 'ordisurf' function implemented in the 'vegan' package. This function fits an environmental surface using thinplate splines in a Generalized Additive Model (GAM; Wood 2006) and then uses results from the GAM to predict and plot the surface on a regularized grid (Oksanen *et al.* 2013). GAM automatically selects the degree of smoothing by cross-validation. In the GAM equation the environmental variable is the dependent variable, and is equal to the sum of the products of the thinplate spline, and the first and second dimensions of the ordination results, respectively. As nectar variables were not normally distributed, to fit the GAM we assumed a Gamma family with log as link function and REML as method. Although maximum, minimum and mean total sugar concentration could be significantly fitted into some ordinations, we just represented the maximum, as the three variables followed a similar pattern (results not shown).

All analyses were computed in the R statistical computing framework version 3.1.1 (R Development Core Team 2014, http://www.cran.r-project.org) and SAS 9.1.3 (SAS Institute, Cary, NC, USA).

RESULTS

Variation in nectar chemical traits

Significant differences in nectar features were found between plant species and families (Table 1; see test values in Supplementary Table S3). Plant families and plant species within families explained between 45.3% (pH) and 87.2% (relative percentage of glucose) of the total variance. For pH, and the relative percentages of the three main sugars, plant species accounted for a statistically significant fraction of the total variance, explaining >40% of the explained variance ($Z = 1.91$, 2.35, 2.81 and 2.22 for nectar pH, and the relative percentages of glucose, fructose and sucrose, respectively). Variance in total sugar concentration, on the other hand, was mainly the result of differences between plant families, which accounted for 86.5% of the explained variance.

Phenotypic variation within yeast species

Isolates from the two yeast species explored a broad phenotypic landscape, as shown by their disparity in the nMDS ordinations

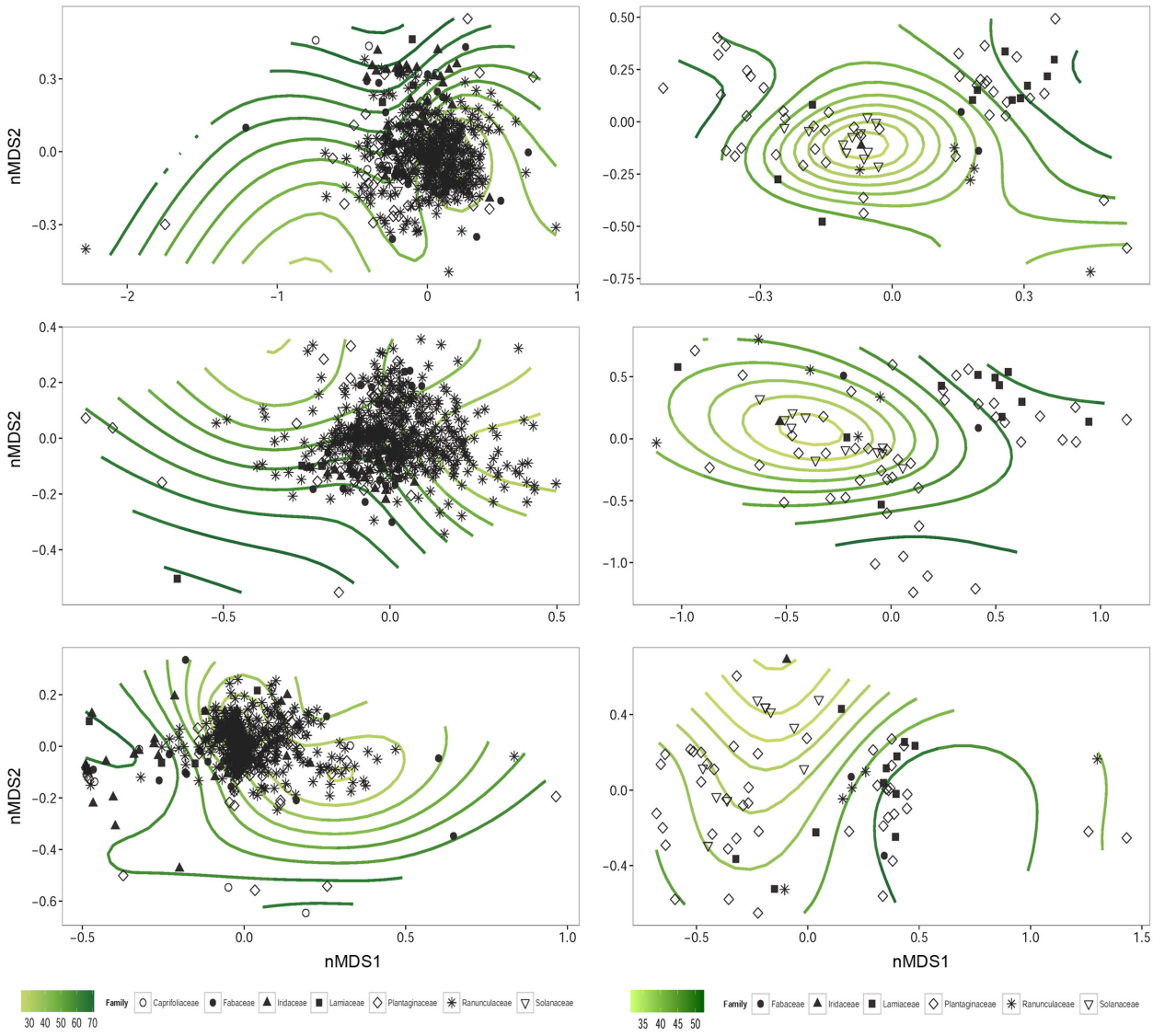

Figure 1. nMDS ordination plots of *M. reukaufii* (left panel) and *M. gruessii* (right panel) isolates. Symbol shapes indicate the plant family from which each isolate was recovered. The gradient of the maximum total sugar concentration is fitted into the ordination diagrams using thinplate splines and is indicated by the green contour lines. Upper panel: all tests. The fit statistics were: R^2(adj) = 0.35 (left) and 0.31 (right), $P < 0.0001$. Middle panel: carbon sources. R^2(adj) = 0.23 and 0.23, $P < 0.0001$, for the left and right figure, respectively. Bottom panel: inhibitors. Fit statistics were R^2(adj) = 0.30 and 0.15, $P < 0.0001$ and $P = 0.001$, for the left and right figure, respectively.

(Fig. 1). The stress values of the nMDS ordinations were similar and below 0.20 for all ordinations.

For *M. reukaufii*, the overall phenotypic profile of the isolates depended significantly on the origin of the sample, measured either by plant family (Pseudo-$F = 11.67$, df = 6, $P = 0.001$) or by plant species (Pseudo-$F = 7.39$, df = 12, $P = 0.001$). These differences were still significant when the *H. foetidus* observations were removed ($n = 212$, Pseudo-$F = 2.02$, df = 6, $P = 0.017$ and Pseudo-$F = 3.76$, df = 11, $P = 0.001$ for plant family and plant species, respectively). Phenotypic variation among *H. foetidus* isolates was partially explained by population of origin and collection date (results not shown). Analyses for every parameter type separately further showed significant differences in the phenotypic profile of isolates from different hosts (Table 2). Only in the case of hydrolysis, pH and vitamin supplementation was there no significant effect of plant family or species on the phenotypic profile (Table 2).

Only a significant effect of host plant species was found in *M. gruessii* for their response to nitrogen sources parameters (Table 2). However, we found a significant effect of the plant family of origin in the overall phenotypic profile (Pseudo-$F = 2.51$, df = 5, $P = 0.05$), and also for carbon sources and inhibitors (Table 2).

Importance of nectar features as environmental constraints

The forward selection procedure showed that for both yeast species, the total sugar concentration in nectar (maximum values per plant species) explained the largest portion of the variance in the overall phenotypic profile of the investigated strains. In both cases, the effect of sugar concentration was highly significant (Pseudo-$F = 29.43$ and 4.35, $P = 0.01$ for *M. reukaufii* and *M. gruessii*, respectively). Another nectar trait that

Table 2. Intraspecific differences in test performance within the studied yeast species (*M. reukaufii* and *M. gruessii*), according to the environmental origin of these species.

Category (no. of parameters tested)	*M. reukaufii*				*M. gruessii*			
	Plant family (df = 6)		Plant species (df = 18)		Plant family (df = 5)		Plant species (df = 3)	
	Pseudo-F	P-value	Pseudo-F	P	Pseudo-F	P-value	Pseudo-F	P-value
Carbon sources (27)	7.32	0.001	5.86	0.001	2.22	0.012	1.48	0.070
Inhibitors (8)	11.74	0.001	7.42	0.001	2.51	0.048	1.56	0.479
Various[a] (4)	1.62	0.647	1.14	0.906	1.38	0.634	4.80	0.068
Low water activity (4)	3.45	0.033	4.26	0.002	0.56	0.447	0.85	0.240
Nitrogen sources (3)	6.75	0.007	8.82	0.001	1.63	0.559	3.31	0.009

For these analyses, phenotypic tests were grouped into five main categories.
[a]Comprises alkaline pH, vitamin, and hydrolysis (see Supplementary Table S2).

Table 3. Pseudo-*F* values of relevant nectar variables selected by additive RDA models ($P < 0.05$) explaining variance at every parameter type, per yeast species.

Parameter type per yeast species	Min total sugar conc	Mean total sugar conc	Max total sugar conc	Min pH	Mean pH	Max pH	% Sucrose	% Fructose	% Glucose	Sum of relevant variables	% variance explained
M. reukaufii											
Overall		3.81	**29.43**					2.68	3.40	**9.93**	6.60%
Carbon sources			**4.85**	**6.77**	**31.64**			**6.42**	**9.90**	**11.63**	9.15%
Inhibitors		3.82	**29.46**				**2.32**	3.04		**6.68**	6.68%
Various			**13.91**							**13.91**	2.40%
M. gruessii											
Overall		3.33	**4.35**					**5.86**		**4.72**	16.84%
Carbon sources		3.33	3.47					**5.74**		**14.36**	14.36%
Inhibitors		4.15	**4.76**					**4.61**		**16.84**	16.84%
Nitrogen		3.43				2.89	3.70			1.95	7.69%
Various	2.62							3.60	2.85	**2.35**	9.16%

Bold fonts reveal those tests with $P < 0.01$.
Zero variables were selected in *M. reukaufii* nitrogen parameters.

significantly explained the overall phenotypic profile within both yeast species was the percentage of fructose in nectar (Table 3). The final model showed that in both yeast species the overall phenotypic profile was significantly related to the maximum total sugar concentration and mean fructose content (Pseudo-*F* values for the overall model: 16.05 and 5.25 for *M. reukaufii* and *M. gruessii*, respectively, $P = 0.005$). Maximum total sugar concentration and relative fructose content also significantly explained intraspecific variance in carbon sources and inhibitors for the two yeast species (Table 3). For nitrogen sources, low water activity and hydrolysis parameters, we did not find any common set of nectar features successfully explaining the phenotypic variance encountered within the two yeast species. While nectar pH explained a significant part of the variance in growth performance of *M. reukaufii* in different carbon sources and low water activity media, the mean relative percentage of glucose significantly explained differences in performance for the overall phenotypic tests, carbon sources and inhibitors. No such relationships were observed for *M. gruessii*.

Fitting significant nectar variables into the phenotypic nMDS ordinations (overall versus by parameter type) confirmed the importance of nectar traits in explaining the phenotypic variation of the nectar isolates of the two yeast species. Graphical representations indicate that the main influence of nectar variables was along nMDS axis 1 for *M. gruessii* and along axis 2 for *M. reukaufii* ordinations (Figs 1 and 2). In *M. gruessii*, isolates located at the right hand side of the graph were from plants with high total sugar concentration. For *M. reukaufii*, strains isolated from plants with high sugar concentration values were located at the lower side of the graph (Fig. 1). Similar trends were observed for sugar composition. For both species, relative fructose content was significantly related to growth performance in all growth media and in carbon source media alone. Isolates located in the upper part of the graph came from plant species with low fructose percentages in their nectar (Fig. 2, upper panel), and the most distant phenotypes regarding carbon sources parameters were isolated from plant species with the most extreme values of fructose concentrations (Fig. 2, middle panel). The opposite trend was obtained in the case of inhibitor ordination in *M. reukaufii* (Fig. 2, bottom panel).

DISCUSSION

Intraspecific variation in phenotypic profile

In this study, we used a large set of physiological tests to characterize intraspecific variation in the phenotypic profile of two common nectarivorous yeasts. The majority of physiological tests that were used here focused on carbon sources, as nectar is mainly comprised of mono- and disaccharides (Percival 1961; Nicolson 2007). However, because the presence of nectar proteins, organic acids and plant secondary compounds in floral nectar has been interpreted as evidence for a putative defence mechanism against microbes (Adler 2000; Heil 2011), we also tested yeast inhibitors.

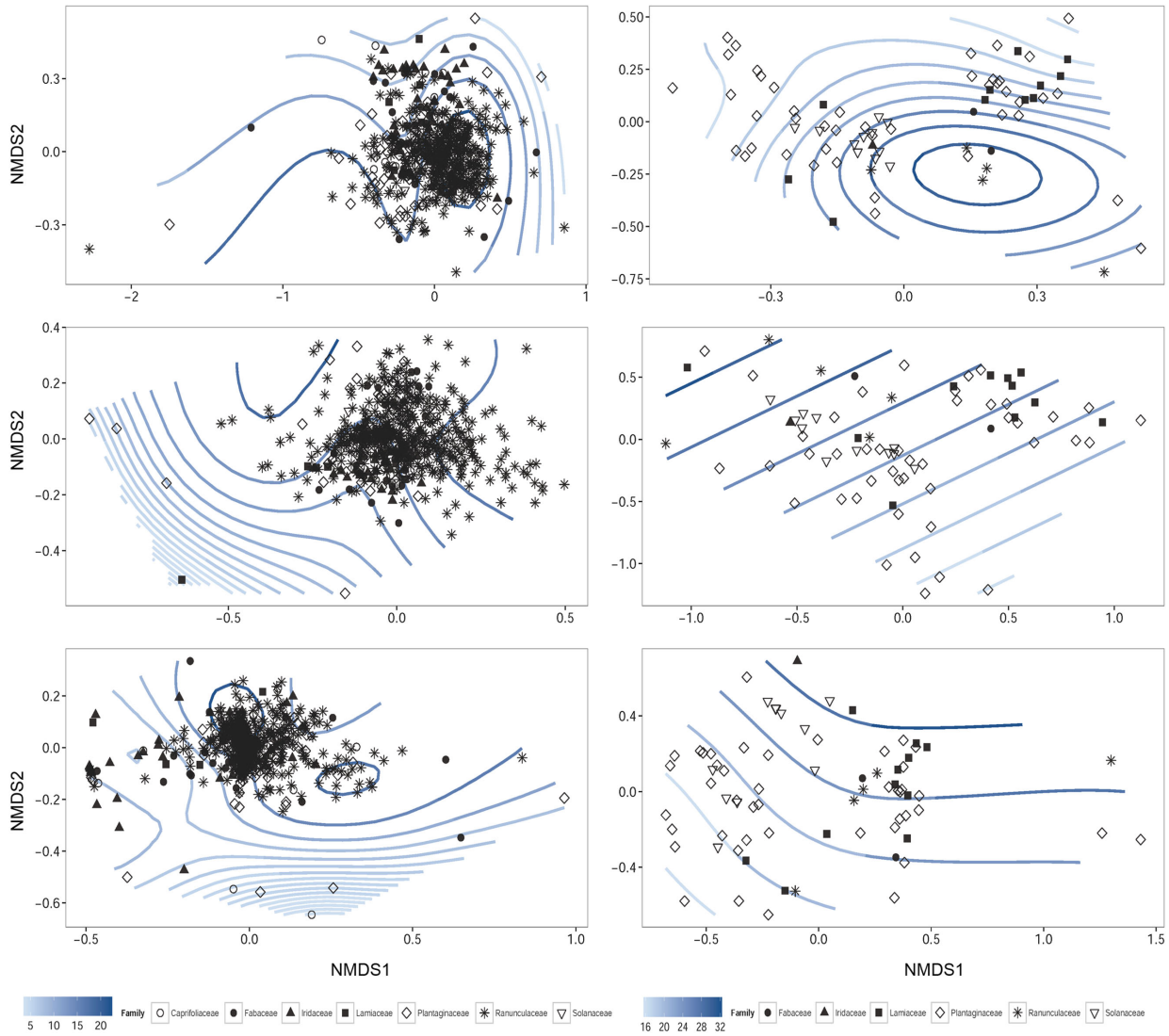

Figure 2. nMDS ordination plots of *M. reukaufii* (left panel) and *M. gruessii* (right panel) isolates. Symbol shapes indicate the plant family from which each isolate was recovered. The gradient of the mean relative percentage of fructose in nectar is fitted into the ordination diagrams using thinplate splines and is indicated by the blue contour lines. Upper panel: all tests. The fit statistics were: R^2(adj) = 0.12 (left) and 0.26 (right), $P < 0.0001$. Middle panel: carbon sources. R^2(adj) = 0.15 and 0.14, $P < 0.0001$ and $P = 0.04$, for the left and right figure, respectively. Bottom panel: inhibitors. Fit statistics were R^2(adj) = 0.10 ($P < 0.0001$) and 0.02, $P = 0.20$, for the left and right figure, respectively.

In *M. gruessii*, wide intraspecific phenotypic variation was observed in a sample of 76 isolates subjected to the physiological tests. Phenotypic variation involved heterogeneity in several test categories, mainly including growth responses to carbon sources and inhibitors. Similar results were found for *M. reukaufii*, for which the sample size was much larger (570 tested isolates). These two species are found in a large proportion of nectaries in several plant species in south-eastern Spain. Differences in sample size are due to the more widespread distribution and larger population sizes of *M. reukaufii* in nectaries in our study area (M.I. Pozo *et al.*, unpublished results). Due to unequal sample size, however, tests have been run separately for each yeast species, and the generalizations that are made throughout the text by using the results of the two yeast species must be treated with caution. Interestingly, our results are in agreement with previous studies on the phenotypic profile of industrial yeast strains (*Saccharomyces*) isolated from different environments, such as wineries and breweries (Salinas *et al.* 2010; Camarasa *et al.* 2012; Barbosa *et al.* 2014; Mukherjee *et al.* 2014). As reported by these studies, we also found that isolates that originate from the same or similar sources tend to show more similar phenotypes. It is challenging to compare our results with previous research on natural populations of non-conventional yeasts, as work on phenotypic features of these species has mainly focused on a few isolates, and intraspecific variability has been generally obliterated by averaging data with the purpose of obtaining diagnostic features for species delimitation (Barnett 1977; Barnett 2004; Kurtzman, Fell and Boekhout 2011). Moreover, growth rates used to be computed on a qualitative scale.

Plant species produce nectars with different quality as yeast substrate

For this study, *M. gruessii* and *M. reukaufii* were isolated from the flowers of different insect-pollinated plant species belonging to six and seven different plant families, respectively. It is already

known that nectar sugar concentration and composition are to a large extent genetically determined (Nicolson and Thornburg 2007). Our results confirmed that nectar features varied widely across plant species. More specifically, we have found that plant species, and even plant families, might be characterized by a given range of nectar sugar concentration, although this feature is also subject to the effects of environmental variation (Corbet *et al.* 1979). Further, all the plant species surveyed tended to produce sucrose-rich nectar, but they differed in the relative amounts of sucrose, fructose and glucose. Although nectar pH has been measured less frequently, it has been shown that it can vary greatly between the plant species involved, ranging between 3 and 10 across plant species (Nicolson and Thornburg 2007). Although some extreme values might be due to the action of nectar-living microbes (Peay, Belisle and Fukami 2012), we have observed a similarly broad pH range in pristine nectar (pH 4–9; see also Herrera 2014). This variation may arise from variable concentrations in nectar of minor solutes such as organic acids or nectar proteins (Herrera 2014).

The nature of the carbon sources available, along with pH and water activity in the yeast environment are known to influence yeast growth (Kurtzman, Fell and Boekhout 2011). Variation in nectar chemical traits may therefore have given rise to specific adaptations and consequently may explain the pervasive effect of host plant species on the phenotypic landscape explored by the two studied yeast species. Recently, Herrera (2014) studied the growth exhibited by individual strains of *M. reukaufii* inoculated in pristine nectar of different plant species, and concluded that different nectars vary in their quality as substrate for yeast growth. Interestingly, *H. foetidus* nectar represented the most favourable growth medium for all strains tested. This plant species produces large amounts of 40% concentrated—on average—and sucrose-rich nectar, in which small amounts of protoanemonin are present (C.M. Herrera, unpublished results). In our case, strains that were isolated from this plant species generally tended to cluster around a similar phenotype. According to Herrera (2014), floral nectar from *Digitalis* (Plantaginaceae) and *Lonicera* (Caprifoliaceae) were the most limiting habitats for yeast growth. Also in our study, Plantaginaceae nectar provided the most dissimilar phenotypes for the two yeast species. Other factors may also explain differences in nectar quality as a growth substrate for yeasts. For instance, the nitrogen content in nectar is very low, and consequentially nectar amino acid content and composition might impact yeast survival in nectar decisively. When yeasts were present, the concentration of several amino acids significantly decreased in *Mimulus aurantiacus* floral nectar (Peay, Belisle and Fukami 2012). In addition, the presence of secondary compounds might also affect yeast growth (Adler 2000; Pozo, Lievens and Jacquemyn 2014), and therefore they might be also investigated regarding their potential at driving the microevolutionary forces in nectar microbes.

Phenotypic traits and nectar chemistry

Total sugar concentration and the relative percentage of fructose in nectar were the most relevant nectar features explaining dissimilarities in phenotypes among the vast collection of nectar samples that were screened. Even if we remove a large proportion of the total sample, i.e. the *M. reukaufii* isolates coming from *H. foetidus*, we get consistent results in redundancy analysis and subsequent variable selection analyses (unpublished results). Therefore, we are confident that those findings are not the result of statistical artefacts. These results are in line with findings of Herrera, Pozo and Bazaga (2012), who showed that the growth of *M. reukaufii* isolates decreased above 40% sugar (w/w), although some isolates still showed measurable growth at 50–55% (w/w). The mean value of the total sugar concentration obtained here for the majority of plant species (<45%) still represents a favourable medium for the growth of these two species (Pozo, Lachance and Herrera 2012). Maximum values of total sugar concentration might thus be considered the main constraint on yeast growth in the nectar of several plant species, as the maximum concentration recorded, often >55%, represents a habitat with a low water activity (Lievens *et al.* 2015). Regarding carbon sources, Herrera, Pozo and Bazaga (2012) also demonstrated that *Metschnikowia* shows a higher growth rate using sucrose as the carbon source compared with its monosaccharides glucose and fructose. Moreover, fructose is the sugar for which those yeasts showed by far a slower and weaker growth (Herrera, Garcia and Perez 2008). The fact that *H. foetidus* nectar was found to the most favourable habitat for yeast growth also agrees with this finding, as *H. foetidus* nectar consists of a 40% concentrated solution of almost pure sucrose. The effect of nectar pH on nectar microbes was first suggested by Brysch-Herzberg (2004), who aimed at explaining the species composition of nectar yeast communities occurring in temperate Western European regions. Although species differed in optimal pH, the optimal range for Saccharomycetales, such as *Metschnikowia*, was found to vary from slightly acid to neutral, depending on the temperature and the presence of oxygen. Unfortunately, there is a paucity of literature on the physiological basis of a preferred pH range for yeasts, but a plausible explanation is that it is due to the optimum pH value for activity of plasma membrane-bound proteins, including enzymes and transport proteins (Narendranath and Power 2005). In the particular case of *M. reukaufii* and *M. gruessii*, it has been shown that neither low (4) nor high (9) pH values seriously affect the growth of these organisms (M.I. Pozo *et al.*, unpublished results). Likewise, we did not find any variation in this feature to be relevant for explaining intraspecific variation in yeast phenotype. We have consistently found that the phenotypic variation exhibited by nectar yeasts regarding nitrogen sources can be significantly explained by the plant species from which those isolates were retrieved. Interestingly, none of our nectar features could explain this variation in the particular case of *M. reukauffii*. In *M. gruessii*, we could explain ~8% of the phenotypic variation regarding nitrogen sources by combining the effect of pH and sugar concentration. We hypothesize that the natural variation in total amino acid concentration and composition in pristine nectar among plant species could help to explain phenotypic dissimilarities in nectar yeasts (Baker and Baker 1983), and we therefore advise the inclusion of this nectar feature for future research.

Mechanisms underlying phenotypic variation

We found that the two yeast species studied were able to live and proliferate under highly divergent conditions, represented by the gradient of pristine nectar conditions of a group of host plant species that flower, with a few exceptions, in the same period (May–June). *Metschnikowia* relies on foraging insects to be vectored from one flower to another, but individual nectaries represent the main reservoir of cells. Given the patchy and ephemeral nature of nectaries as habitat, those yeast communities function as metacommunities. In the wild, clonal lineages from these two yeast species explore different niches during their life cycle (nectar from different plant species). Such a life cycle in turn imposes additional pressure on the ability of these microorganisms to cope with a broad range of nectar environments and thus

requires the ability to adapt rapidly to different nectar conditions. Consequently, it might be highly favourable for these organisms to keep broad ecological niches and show high levels of phenotypic plasticity (Roughgarden 1972; Sultan and Spencer 2002; Baythavong 2011).

However, habitat heterogeneity may drive more permanent adaptive responses in yeasts, with higher evolutionary potential. Although clonal reproduction is the dominant reproduction mode of these two yeast species, they possess a high genotypic diversity. Moreover, genotypes are not randomly sorted in nature, and it has therefore been shown that different genotypes are favoured by the action of diversifying selection on contrasting microenvironments (Herrera, Pozo and Bazaga 2011; Herrera, Pozo and Bazaga 2014). A link between genotype and isolation source was also observed for *Brettanonmyces bruxellensis*, a yeast species important to both the brewing and wine industry (Crauwels *et al*. 2014). Furthermore, epigenetic changes that occur as a result of DNA methylation have been shown to contribute to population niche width by enhancing phenotypic plasticity. In *M. reukaufii*, genome-wide DNA methylation patterns underlaid the ability of this fugitive species to exploit a broad resource range in its heterogeneous and patchy environment, mimicked experimentally by increasing concentrations of sucrose, glucose and fructose (Herrera, Pozo and Bazaga 2012).

Overall, our results highlight the importance of assessing the degree of intraspecific variation in order to obtain more realistic information on the phenotypic profile exhibited by yeast species. This wide intraspecific variance might be explained by the natural origin of the isolates, in this case represented by nectar from different plant host species. We have shown that the variation exhibited across plant species in total sugar concentration and sugar composition (especially the relative amount of fructose) relevantly accounted for the differences found in the yeast phenotypes according to their origin. We thus hypothesize that both nectar properties and the plant host species of origin might predict the yeast phenotypes that will occur in wild populations of nectar yeasts.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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REFERENCES

Abramoff MD, Magalhaes PJ, Ram SJ. Image processing with ImageJ. *Biophotonics Int* 2004;**11**:36–43.

Adler LS. The ecological significance of toxic nectar. *Oikos* 2000;**91**:409–20.

Álvarez-Pérez S, Herrera CM, de Vega C. Zooming-in on floral nectar: a first exploration of nectar-associated bacteria in wild plant communities. *FEMS Microbiol Ecol* 2013;**80**:591–602.

Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 2001;**26**:32–46.

Baker HG, Baker I. Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ (eds) *Handbook of Experimental Pollination Biology*. New York: Van Nostrand Reinhold, 1983, 117–41.

Barbosa C, Lage Pc, Vilela A, *et al*. Phenotypic and metabolic traits of commercial *Saccharomyces cerevisiae* yeasts. *AMB Express* 2014;**4**:1–14.

Barnett JA. Nutritional tests in yeast systematics. *J Gen Microbiol* 1977;**99**:183–90.

Barnett JA. A history of research on yeasts 8: taxonomy. *Yeast* 2004;**21**:1141–93.

Baythavong BS. Linking the spatial scale of environmental variation and the evolution of phenotypic plasticity: selection favors adaptive plasticity in fine-grained environments. *Am Nat* 2011;**178**:75–87.

Belisle M, Peay KG, Fukami T. Flowers as islands: spatial distribution of nectar-inhabiting microfungi among plants of *Mimulus aurantiacus*, a hummingbird-pollinated shrub. *Microb Ecol* 2012;**63**:711–8.

Brysch-Herzberg M. Ecology of yeasts in plant–bumblebee mutualism in Central Europe. *FEMS Microbiol Ecol* 2004;**50**:87–100.

Camarasa C, Sanchez I, Brial P, *et al*. Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: evidence for origin-dependent metabolic traits. *PLoS One* 2012;**6**:e25147.

Clarke KR, Warwick RM. *Change in Marine Communities: An Approach to Statistical Analysis and Interpretation*, 2nd edn. Plymouth: PRIMER-E, 2001.

Corbet SA, Willmer PG, Beament JWL, *et al*. Post-secretory determinants of sugar concentration in nectar. *Plant Cell Environ* 1979;**2**:293–308.

Crauwels S, Zhu B, Steensels J, *et al*. Assessing genetic diversity in *Brettanomyces* yeasts using DNA fingerprinting and whole genome sequencing. *Appl Environ Microbiol* 2014;**80**:4398–413.

Dafni A. *Pollination Ecology: A Practical Approach*. Oxford: IRL Press (Oxford University Press), 1992.

de Vega C, Herrera CM. Microorganisms transported by ants induce changes in floral nectar composition of an ant-pollinated plant. *Am J Bot* 2013;**100**:792–800.

de Vega C, Herrera CM, Johnson SD. Yeasts in floral nectar of some South African plants: quantification and associations with pollinator type and sugar concentration. *South Afr J Bot* 2009;**75**:798–806.

Dittmar JC, Reid RJD, Rothstein R. ScreenMill: a freely available software suite for growth measurement, analysis and visualization of high-throughput screen data. *BMC Bioinformatics* 2010;**11**.

Fridman S, Izhaki I, Gerchman Y, Halpern M. Bacterial communities in floral nectar. *Environ Microbiol Rep* 2012;**4**:97–104.

Galetto L, Bernardello G. Rewards in flowers: nectar. In: Dafni A, Kevan PG, Husband BC (eds) *Practical Pollination Biology*. Cambridge, Ontario: Enviroquest, Ltd, 2005, 261–313.

Heil M. Nectar: generation, regulation, and ecological functions. *Trends Plant Sci* 2011;**16**:191–200.

Herrera CM. Population growth of the floricolous yeast *Metschnikowia reukaufii*: effects of nectar host, yeast genotype, and host × genotype interaction. *FEMS Microbiol Ecol* 2014;**88**:250–7.

Herrera CM, Canto A, Pozo MI, *et al*. Inhospitable sweetness: nectar filtering of pollinator-borne inocula leads to

impoverished, phylogenetically clustered yeast communities. *Proc R Soc B: Biol Sci* 2010;**277**:747–54.

Herrera CM, de Vega C, Canto A, *et al*. Yeasts in floral nectar: a quantitative survey. *Ann Bot* 2009;**103**:1415–23.

Herrera CM, Garcia IM, Perez R. Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants. *Ecology* 2008;**89**:2369–76.

Herrera CM, Pérez R, Alonso C. Extreme intraplant variation in nectar sugar composition in an insect-pollinated perennial herb. *Am J Bot* 2006;**93**:575–81.

Herrera CM, Pozo MI, Bazaga P. Clonality, genetic diversity and support for the diversifying selection hypothesis in natural populations of a flower-living yeast. *Mol Ecol* 2011;**20**:4395–407.

Herrera CM, Pozo MI, Bazaga P. Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. *Mol Ecol* 2012;**21**:2602–16.

Herrera CM, Pozo MI, Bazaga P. Non-random genotype distribution among floral hosts contributes to local and regional genetic diversity in the nectar-living yeast *Metschnikowia reukaufii*. *FEMS Microbiol Ecol* 2014;**87**:568–75.

Kurtzman C, Fell J, Boekhout T. *The Yeasts: A Taxonomic Study*, 5th edn. Amsterdam: Elsevier, 2011.

Lachance MA. *Metschnikowia* Kamienski (1899). In: Kurtzman C, Fell J, Boekhout T (eds) *The Yeasts, a Taxonomic Study*, 5th edn. Amsterdam: Elsevier, 2011, 575–620.

Lachance MA, Starmer W. Ecology and yeasts. In: Kurtzman C, Fell J (eds) *The Yeasts, a Taxonomy Study*, 4th edn. Amsterdam: Elsevier, 1998, 21–30.

Lievens B, Hallsworth JE, Pozo MI, *et al*. Microbiology of sugar-rich environments: diversity, ecology and system constraints. *Environ Microbiol*, 2015;**17**:278–98.

Majetic CJ, Levin DA, Raguso RA. Divergence in floral scent profiles among and within cultivated species of *Phlox*. *Sci Hort* 2014;**172**:285–91.

Mukherjee V, Steensels J, Lievens B, *et al*. Phenotypic evaluation of natural and industrial *Saccharomyces* yeasts for different traits desirable in industrial bioethanol production. *Appl Microbiol Biotechnol* 2014;**98**:9483–98.

Napier KR, McWhorter TJ, Nicolson SW, *et al*. Sugar preferences of avian nectarivores are correlated with intestinal sucrase activity. *Physiol Biochem Zool* 2013;**86**:499–514.

Narendranath NV, Power R. Relationship between pH and medium dissolved solids in terms of growth and metabolism of lactobacilli and *Saccharomyces cerevisiae* during ethanol production. *Appl Environ Microbiol* 2005;**71**:2239–43.

Nicolson SW. Nectar consumers. In: Nicolson SW, Nepi M, Pacini E (eds) *Nectaries and Nectar*. Dordrecht, The Netherlands: Springer, 2007, 289–42.

Nicolson SW, Thornburg RW. Nectar chemistry. In: Nicolson SW, Nepi M, Pacini E (eds) *Nectaries and Nectar*. Dordrecht, The Netherlands: Springer, 2007, 215–64.

O'Donnell K. *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*. Wallingford: CAB International, 1993, 225–33.

Oksanen J, Blanchet FG, Kindt R, *et al*. Package 'vegan'. *Community Ecol Packag, version 2.0-7*, 2013.

Peay KG, Belisle M, Fukami T. Phylogenetic relatedness predicts priority effects in nectar yeast communities. *Proc R Soc B:Biol Sci* 2012;**279**:5066–6.

Percival MS. Types of nectar in angiosperms. *New Phytol* 1961;**60**:235–81.

Petanidou T. Sugars in Mediterranean floral nectars: an ecological and evolutionary approach. *J Chem Ecol* 2005;**31**:1065–88.

Petanidou T, Van Laere A, Ellis WN, *et al*. What shapes amino acid and sugar composition in Mediterranean floral nectars? *Oikos* 2006;**115**:155–69.

Pozo MI, Herrera CM, Bazaga P. Species richness of yeast communities in floral nectar of Southern Spanish plants. *Microb Ecol* 2011;**61**:82–91.

Pozo MI, Lachance MA, Herrera CM. Nectar yeasts of two southern Spanish plants: the roles of immigration and physiological traits in community assembly. *FEMS Microbiol Ecol* 2012;**80**:281–93.

Pozo MI, Lievens B, Jacquemyn H. Impact of microorganisms on nectar chemistry, pollinator attraction and plant fitness. In: Peck RL (ed). *Nectar: Production, Chemical Composition and Benefits to Animals and Plants*. New York: Nova Science Publishers, Inc., 2014.

Roughgarden J. Evolution of niche width. *Am Nat* 1972;**106**:683–718.

Salinas F, Cubillos FA, Soto D, *et al*. The genetic basis of natural variation in oenological traits in Saccharomyces cerevisiae. *PLoS One* 2010;**7**:e49640.

Simpson BB, Neff JL. Evolution and diversity of floral rewards. In: Jones CE, Little RJ (eds) *Handbook of Experimental Pollination Biology*. New York: Van Nostrand Reinhold, 1983, 142–59.

Sultan SE, Spencer HG. Metapopulation structure favors plasticity over local adaptation. *Am Nat* 2002;**160**:271–83.

Vergauwen R, Van den Ende W, Van Laere A. The role of fructan in flowering of *Campanula rapunculoides*. *J Exp Bot* 2000;**51**:1261–6.

Wood S. *Generalized Additive Models: An Introduction With R*. Florida: Chapman Hall/CRC Press, 2006.